Molecular Systematics of Old World Stripe-Necked Turtles (Testudines: *Mauremys*)

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Abstract. - Nine extant species of *Mauremys* (including *Ocadia* and *Chinemys*) represent a geographically widespread yet morphologically and ecologically conservative group of batagurid turtles. Here we examine the evolutionary relationships of *Mauremys* using 1539 base pairs of mitochondrial DNA encoding portions of COI, ND4, and three adjacent tRNA genes. These data contain 246 parsimony informative characters that we use to erect hypotheses of relationships for *Mauremys*. Both maximum parsimony and Bayesian methods suggest that *Mauremys japonica, M. sinensis, M. nigricans,* and *M. reevesii* form a well-supported monophyletic clade, as do *M. mutica* and *M. annamensis.* Furthermore, our analyses show that *M. mutica* is paraphyletic with respect to *M. annamensis.* The western taxa *M. leprosa, M. caspica,* and *M. rivulata* remain problematic and do not form a monophyletic group sister to the Asian taxa. Nevertheless, an east-west biogeographic hypothesis cannot be discounted with our molecular genetic data.

Key words. - Turtles, Bataguridae, *Mauremys,* molecular phylogenetics, mitochondrial DNA

Introduction

The Old World turtle genus *Mauremys* is represented by morphologically and ecologically conservative species that are diagnosed by a rigid plastron and a striped head and neck. These semi-aquatic, batagurid (= geoemydid, see Joyce et al., in press) turtles occupy lotic and lentic environments in both forested and arid habitats throughout Asia and the Mediterranean.

The genus contains some of the most commercially important freshwater turtles in Asia. For example, *M. mutica* is one of the most commonly reared and highly traded chelonians in Asia (Lau and Shi, 2000). Other *Mauremys* species have been at the center of a conservation and systematics controversy. In fact, two newly described *Mauremys* may be polyphyletic hybrids (Parham et al., 2001; Wink et al., 2001; Spinks et al., 2004).

Given the mounting conservation interest in the turtle fauna of Asia (van Dijk et al., 2000), understanding the extant diversity and phylogenetic relationships among the Bataguridae are areas of active research (Wu et al., 1999; Honda et al., 2002b; Barth et al., 2004; Spinks et al., 2004). The genus *Mauremys* has received particular attention because of this recent conservation crisis and taxonomic confusion. The first examination of evolutionary relationships within *Mauremys* was a morphological treatment of the genus based on shell and scute measurements (Iverson and McCord, 1994). Consistent with the disjunct distribution of *Mauremys,* Iverson and McCord (1994) suggested that East Asian taxa form a monophyletic group, sister to a Mediterranean and Middle Eastern clade. A subsequent study used 12S and 16S ribosomal genes to resolve the phylogenetic relationships among species of *Mauremys* (Honda et al., 2002a). In contrast to the east-west hypothesis of Iverson and McCord (1994), Honda et al. (2002a) suggested that the deepest phylogenetic splits within *Mauremys* occur between Asian taxa. The ribosomal mtDNA data also cast doubt on the monophyly of traditional *Mauremys* by including the east Asian species, *Chinemys reevesii,* as the sister taxon to *M. japonica.* Two recent studies examined more extensive sequence data, predominantly cyt *b* mtDNA, as well as a more comprehensive sampling of batagurids (Barth et al., 2004; Spinks et al., 2004). Both studies firmly established the placement of *Mauremys* within the Bataguridae and show that the *Chinemys* and *Ocadia* are phylogenetically nested within *Mauremys* (Barth et al., 2004; Spinks et al., 2004). Barth et al. (2004) offer two possible solutions to reconcile the paraphyly of *Mauremys:* 1) split the species of *Mauremys* into four genera; 2) lump *Chinemys* and *Ocadia* into an expanded *Mauremys.* While Barth et al. (2004) refrain from a taxonomic decision, Spinks et al. (2004) adopt an expanded *Mauremys.* We also endorse an inclusive *Mauremys*

because we consider expanding genera to well-supported clades of species functionally preferable to proliferating monotypic genera based on subjective, typological ideas of uniqueness (Feldman and Parham, 2002; Parham and Feldman, 2002; Spinks et al., 2004).

Our objective here is to provide an independent estimate of *Mauremys* phylogeny using different molecular markers from other recent systematic investigations and separate museum voucher specimens (Barth et al., 2004; Spinks et al., 2004). We hope that our data help resolve areas of uncertainty in the emerging consensus on *Mauremys* systematics. In addition, our study will add to the growing body of information on the evolutionary history and diversity of Asia's threatened batagurid fauna (Wu et al., 1999; Honda et al., 2002b; Barth et al., 2004; Spinks et al., 2004).

Materials and methods

Taxon sampling and laboratory protocols. - We obtained liver tissue from 17 museum specimens representing nine currently recognized species of *Mauremys* and three species of *Cuora* (Appendix 1). The nine species of *Mauremys* used in our study include: *M. annamensis, M. caspica, M. japonica, M. leprosa, M. mutica, M. nigricans, M. reevesii, M. rivulata,* and *M. sinensis*. We do not consider "*M. iversoni*", "*M. pritchardi*", "*O. glyphistoma*" or "*O. philippeni*" to be valid taxa because specimens matching these species (all described from the pet trade) are likely hybrids (Parham et al., 2001; Wink et al., 2001; Spinks et al., 2004). In addition, we also excluded "*M. megalocephala*", which is probably a diet-induced variant of *M. reevesii* (Iverson et al., 1989; Barth et al., 2002). However, we do include a "*M. iversoni*"-like hybrid specimen described in Parham and Shi (2001) because mtDNA from this hybrid specimen is demonstrably *Mauremys* (Parham et al., 2001). All vouchers correspond to well-documented reference material and original species descriptions.

We isolated genomic DNA from tissue samples by standard proteinase K digestion and phenol/chloroform purification (Maniatis et al., 1982). We amplified 700 bp of mtDNA encoding a section of COI via PCR (Saiki et al., 1988) using primers HCO-2193 and LCO-1490 (Folmer et al., 1994). We amplified an additional 900 bp region of mtDNA encoding a portion of ND4 and flanking tRNA histidine (tRNA^{his}), serine (tRNA^{ser}), and part of leucine (tRNA^{leu}) using primers ND4 and Leu (Arevalo et al., 1994). We used the following thermal cycle parameters for 50µl amplification reactions: 35 cycles of 1min denature at 94°C, 1min anneal at 45°C (COI) or 52°C (ND4), and 2min extension at 72°C. We purified PCR products using the Wizard Prep Mini Column Purification Kit (Promega, Inc.) and used puri-

fied template in 10µl dideoxy chain-termination reactions (Sanger et al., 1977) using ABI Big Dye chemistry (Applied Biosystems, Inc.) and the primers listed above. Following an isopropanol/ethanol precipitation, we ran cycle-sequenced products on a 4.8% Page Plus (Ameresco) acrylamide gel using an ABI 377 automated sequencer (Applied Biosystems, Inc.). We sequenced all samples in both directions.

Sequence analyses. - We aligned DNA sequences with the program Sequencher™ 4.1 (Gene Codes Corp.), and translated protein coding nucleotide sequences into amino acid sequences using MacClade 4.0 (Maddison and Maddison, 2000). We identified tRNA genes by manually reconstructing their secondary structures using the criteria of Kumazawa and Nishida (1993). We deposited all mitochondrial DNA sequences in GenBank (Appendix 1).

We performed a partition homogeneity test (PH), similar to the incongruence length differences test (ILD; Farris et al., 1994), to determine whether the ND4 and COI data could be combined. We used PAUP* 4.0b10 (Swofford, 2002) to generate a null distribution of length differences using 1000 same-sized, randomly generated partitions from the original data with replacement.

To evaluate base substitution saturation at first, second, and third codon positions, we plotted the uncorrected percent sequence divergence of transitions and transversions versus the corrected maximum likelihood estimates of divergence for each codon position.

Phylogenetic analyses. - We used maximum parsimony (MP; Farris, 1983) and maximum likelihood-based Bayesian (Larget and Simon, 1999) phylogenetic methods to infer evolutionary relationships among batagurid species. We conducted MP analyses in PAUP* and Bayesian analyses with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). We polarized the phylogeny via outgroup comparison (Maddison et al., 1984) using the Asian box turtles *Cuora mouhotii, Cuora picturata,* and *Cuora trifasciata*. Other molecular phylogenetic studies suggest these turtles are appropriate outgroup taxa (Wu et al., 1999; Honda et al., 2002b; Barth et al., 2004; Spinks et al., 2004).

We executed MP analyses with the branch-and-bound search algorithm (Hendy and Penny, 1982) using equally weighted, unordered characters. To assess nodal support, we used the bootstrap resampling method (BP; Felsenstein, 1985) employing 1000 pseudoreplicates of branch-and-bound searches in PAUP*. Additionally, we calculated branch support (DI; Bremer, 1994) for all nodes using the program Tree Rot 2c (Sorenson, 1999).

We performed Bayesian analyses to estimate branch lengths and search for additional tree topologies. To

determine the most appropriate model of DNA substitution for reconstructing *Mauremys* relationships under the Bayesian method, we executed hierarchical likelihood ratio tests (LRT; Felsenstein, 1993; Goldman, 1993; Yang, 1996) in the program Modeltest 3.06 (Posada and Crandall, 1998). Because MrBayes 3.0b4 can perform singular phylogenetic analyses using different models of evolution, we performed two separate LRTs on the two mtDNA regions. The model of nucleotide substitution that best fit the COI data was the HKY model (Hasegawa et al., 1985) in conjunction with Γ (Yang, 1994a; 1994b), and I (Gu et al., 1995), while the slightly less complex HKY + Γ model of DNA evolution best fit the ND4 data. We then performed Bayesian tree searches, allowing separate parameter estimates under the two models of DNA substitution for the COI and ND4 data partitions. We did not specify a topology or nucleotide substitution model parameters *a priori*. We ran Bayesian analyses for 3×10^6 generations using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm with four heated Markov chains per generation, sampling trees every 100 generations. To determine when the Markov chains had converged on stable likelihood values, we plotted the -lnl scores against the number of generations (Huelsenbeck and Ronquist, 2001). We then computed a 50 % majority rule consensus tree after excluding those trees sampled prior to the stable equilibrium. Nodal support is given by the frequency of the recovered clade, which corresponds to the posterior probability of that clade under the given models of sequence evolution (PP; Rannala and Yang, 1996; Huelsenbeck and Ronquist, 2001). Lastly, we performed three Bayesian runs to be sure that independent analyses converged on similar log-likelihood scores (Leaché and Reeder, 2002).

Results

Genetic variation. - Sequences from the protein coding regions appear functional and there are no gene rearrangements in the data (Kumazawa and Nishida, 1995; Kumazawa et al., 1996; Macey and Verma, 1997; Macey et al., 1997). However, ND4 in the batagurids studied here appears truncated relative to that of emydid turtles, which have three additional residues: Phenylalanine, Tyrosine, and Cysteine (Feldman and Parham, 2002). Instead, these batagurids possess a stop codon, followed by a 12 bp stretch of highly polymorphic DNA between ND4 and tRNA[his]. Additionally, tRNA[ser] has a short D-stem, instead of a D-arm replacement loop like that of most metazoan taxa (Kumazawa and Nishida, 1993). This unusual tRNA condition is also seen in emydid turtles (Feldman and Parham, 2002).

The PH test shows that length difference between the sum of the COI and ND4 trees and the combined COI and ND4 trees is not significantly different from the randomly generated test statistic ($P = 0.93$). Therefore, we combined the aligned DNA sequences for subsequent phylogenetic analyses.

Of the 1539 aligned nucleotides, 369 are variable and 246 are parsimony informative. Among ingroup taxa, 289 sites are variable and 205 parsimony informative. Of the 369 variable characters, 60 occur at 1st codon positions, 15 at 2nd positions, 261 at 3rd positions, and 33 in tRNAs. The scatter diagrams are linear and show no evidence of multiple hit problems for transitions or transversions (data not shown).

Phylogenetic relationships. - The branch-and-bound equally weighted MP analysis produces a single most parsimonious tree (L = 661; CI = 0.626; RI = 0.683) that is consistent with the model-based Bayesian analyses (Fig. 1). All three Bayesian analyses converge on the same topology and nearly identical mean log-likelihood values, parameter estimates, and nodal support. Thus we simply present results from the final search. The partitioned HKY + Γ + I and HKY + Γ Bayesian analysis (3×10^6 generations) attains stable log-likelihood values within the first 15,000 generations, but we were conservative and discarded the first 20,000 generations. Because we sampled trees every 100 generations, we discarded the first 200 trees and retained 29,800 Bayesian trees, which we used to generate a 50% majority rule tree, and for which consensus values represent a group's posterior probability (Huelsenbeck and Ronquist, 2001). The summary topology of the nearly 30,000 Bayesian trees (mean -lnl = 5205.5110, σ^2 = 24.5038; mean ti/tv (COI) = 10.8360; σ^2 = 10.6335; mean α (COI) = 0.5479, σ^2 = 0.8874; mean P_{invar} (COI) = 0.4163, σ^2 = 0.0291; mean ti/tv (ND4) = 12.3499; σ^2 = 9.0505; mean α (ND4) = 0.2431, σ^2 = 0.0009) differs from the MP tree in the placement of only one taxon (Fig. 1).

In both analyses, species of *Cuora* unambiguously group to the exclusion of *Mauremys*, (BP = 100%; DI = 19; PP = 100%). *Mauremys japonica* is a member of a clade containing *M. nigricans, M. reevesii* and *M. sinensis* (BP = 100%; DI = 13; PP = 100%), yet relationships among these taxa are not well resolved, as indicated by the low nodal support and conflict between MP and Bayesian reconstructions. The MP tree places *M. sinensis* sister to a group linking *M. japonica, M. nigricans*, and *M. reevesii* (DI = 1), wherein *M. nigricans* and *M. reevesii* form an additional clade (BP = 86%; DI = 5). Alternatively, the Bayesian tree connects *M. japonica* to *M. sinensis* (PP = 59%), sister to the *M. nigricans* the *M. reevesii* clade (PP = 99%). The *M. japonica, M. nigricans, M. reevesii*, and *M. sinensis* clade is sister to a
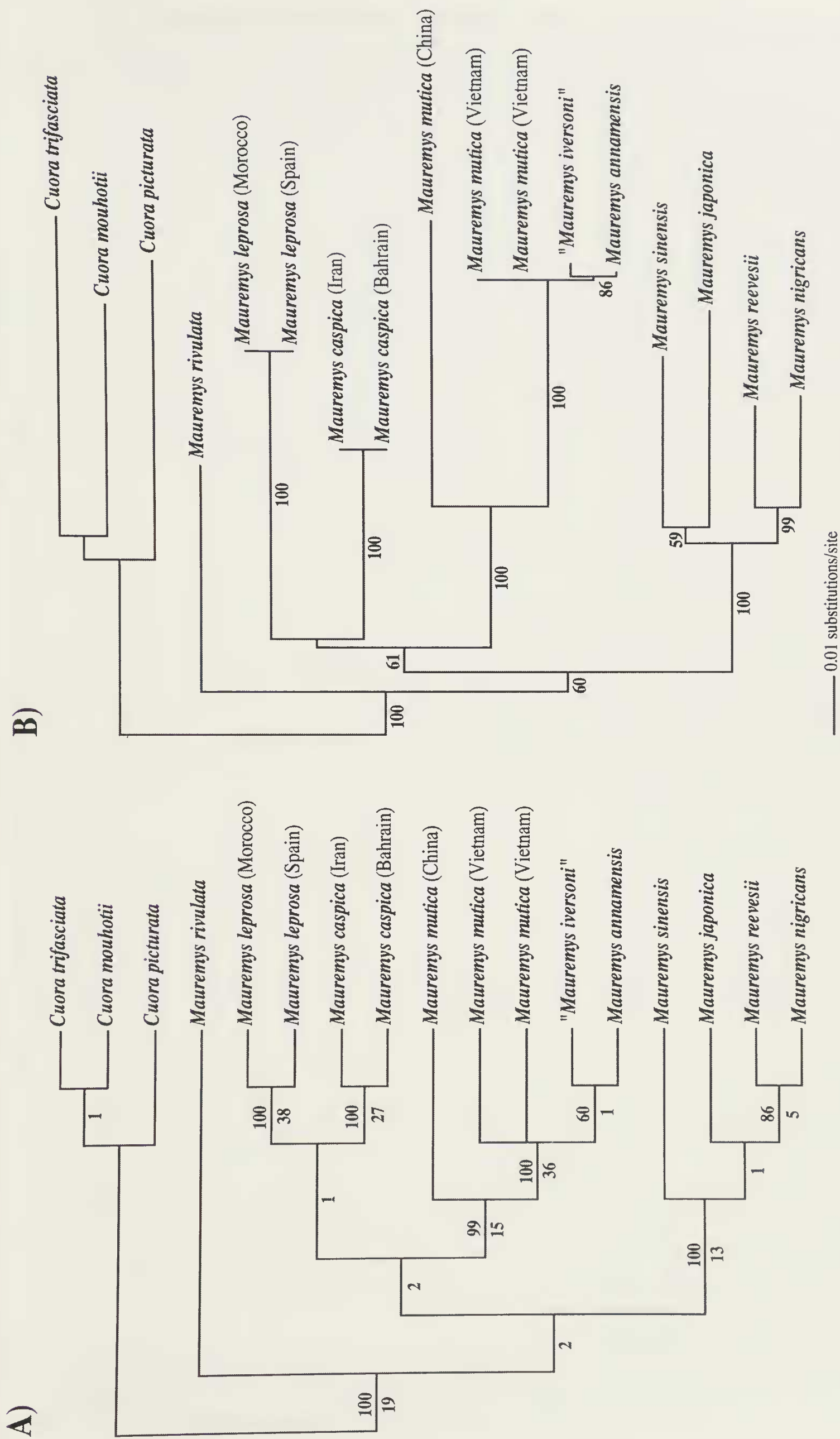
Figure 1. Phylogenetic trees for *Mauremys*. Country of origin given for species with multiple samples; hybrid taxon is in quotes. A) Single most parsimonious tree based on equally weighted characters (L = 661; CI = 0.626; RI = 0.683). Numbers above nodes indicate bootstrap support, those below nodes represent decay indices. B) Bayesian estimate of *Mauremys* phylogeny based on 29,800 trees built under partitioned HKY + Γ + I and HKY + Γ models of DNA evolution (mean -lnI = 5205.5110, σ² = 24.5038; mean ti/tv (COI) = 10.8360; σ² = 10.6335; mean α(COI) = 0.5479, σ² = 0.8874; mean P$_{invar}$ (COI) = 0.4163, σ² = 0.0291; mean ti/tv (ND4) = 12.3499; σ² = 9.0505; mean α (ND4) = 0.2431, σ² = 0.0009). Numbers along nodes represent posterior probability values. Branch lengths drawn proportional to Bayesian estimates of genetic divergence.

Table 1. Pairwise comparisons of mtDNA sequences among Mauremys and related taxa. Note: values above the diagonal indicate uncorrected pairwise differences (%) while those below the diagonal denote HKY + Γ + I sequence divergences (%).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 M. nigricans	-	3.12	7.74	5.66	5.66	7.74	5.07	6.31	6.31	8.26	7.61	7.61	6.18	4.16	8.65	8.71	8.58
2 M. reevesii	3.68	-	7.74	5.33	5.33	7.74	4.75	6.50	6.50	8.06	7.61	7.61	6.18	4.42	8.65	8.39	8.32
3 M. annamensis	11.72	11.72	-	6.89	6.89	0.26	8.65	7.67	7.67	6.31	0.13	0.13	6.83	8.32	8.97	9.30	9.62
4 M. caspica (Iran)	7.56	7.01	9.75	-	0	7.02	6.76	5.92	5.92	7.09	6.76	6.76	5.79	6.24	8.71	8.52	8.52
5 M. caspica (Bahrain)	7.56	7.01	9.75	0	-	7.02	6.76	5.92	5.92	7.09	6.76	6.76	5.79	6.24	8.71	8.52	8.52
6 "M. iversoni"	11.71	11.71	0.26	10.00	10.00	-	8.65	7.67	7.67	6.31	0.26	0.26	6.96	8.32	8.84	9.17	9.49
7 M. japonica	6.63	6.16	13.95	9.78	9.78	13.94	-	8.00	8.00	8.58	8.52	8.52	7.48	4.94	10.14	9.75	9.36
8 M. leprosa (Spain)	8.92	9.28	11.59	7.98	7.98	11.59	12.66	-	0	7.80	7.54	7.54	6.96	6.83	8.45	8.13	8.13
9 M. leprosa (Morocco)	8.92	9.28	11.59	7.98	7.98	11.59	12.66	0	-	7.80	7.54	7.54	6.96	6.83	8.45	8.13	8.13
10 M. mutica (China)	12.83	12.68	8.96	10.34	10.34	8.96	13.95	12.13	12.13	-	6.18	6.18	7.54	7.41	9.69	9.49	9.49
11 M. mutica (Vietnam)	11.45	11.44	0.13	9.51	9.51	0.26	13.64	11.32	11.32	8.72	-	0	6.70	8.19	8.84	9.17	9.49
12 M. mutica (Vietnam)	11.45	11.44	0.13	9.51	9.51	0.26	13.64	11.32	11.32	8.72	0	-	6.70	8.19	8.84	9.17	9.49
13 M. rivulata	8.57	8.65	9.86	7.75	7.75	10.11	11.37	10.15	10.15	11.30	9.61	9.61	-	6.05	8.32	8.00	8.32
14 M. sinensis	5.17	5.62	13.24	8.74	8.74	13.22	6.52	10.09	10.09	11.25	12.94	12.94	8.49	-	9.04	8.39	8.26
15 Cuora picturata	13.92	14.08	14.82	13.80	13.80	14.50	18.11	13.64	13.64	16.55	14.50	14.50	12.99	15.16	-	6.57	7.35
16 Cuora trifasciata	14.29	13.67	15.88	13.56	13.56	15.54	17.35	13.10	13.10	16.33	15.54	15.54	12.48	13.81	9.26	-	6.63
17 Cuora mouhotii	14.33	13.85	16.64	13.90	13.90	16.31	16.55	13.42	13.42	16.55	16.31	16.31	13.51	13.85	11.02	9.72	-

poorly supported *M. mutica*, *M. annamensis*, "*M. iversoni*", *M. leprosa*, and *M. caspica* assemblage (DI = 2; PP = 61%). Within this large group, *M. mutica*, *M. annamensis* and "*M. iversoni*" form a strongly supported clade (BP = 99; DI = 15; PP = 100%). In fact, inclusion of both *M. annamensis* and "*M. iversoni*" render *M. mutica* paraphyletic; two *M. mutica* (ROM 25613, 25614) are more closely related to *M. annamensis* and "*M. iversoni*" than they are to a Chinese *M. mutica* (MVZ 230476) (BP = 100%; DI = 36; PP = 100%). Within this "*mutica* complex", *M. annamensis* and "*M. iversoni*" are weakly allied (BP = 60%; DI = 1; PP = 86). This entire "*mutica* complex" is then sister to a weakly supported *M. leprosa* and *M. caspica* clade (DI = 1). Finally, both MP and Bayesian analyses suggest that *M. rivulata* is sister to a monophyletic clade containing the rest of *Mauremys*, but this phylogenetic arrangement receives almost no statistical support (DI = 2; PP = 60%).

Discussion

Phylogenetic relationships. - Both MP and Bayesian phylogenetic methods show that *M. japonica* is a member of a clade containing *M. nigricans*, *M. reevesii*, and *M. sinensis*, exclusive of other *Mauremys*. The *M. japonica*, *M. nigricans*, *M. reevesii*, and *M. sinensis* clade is joined to a poorly supported *M. mutica*, *M. annamensis*, *M. leprosa*, and *M. caspica* assemblage. Within this grouping, *M. mutica* and *M. annamensis* form a solid clade, congruent with shell and scute data (Iverson and McCord, 1994), other molecular data (Barth et al., 2004; Spinks et al., 2004) but not 12S and 16S mtDNA data (Honda et al., 2002a). Our analyses further suggest that *M. mutica* is paraphyletic. Two *M. mutica* (ROM 25613, 25614) purchased in Vietnam are more closely related to *M. annamensis* than they are to topotypic *M. mutica* (MVZ 230476) from China. We tested the paraphyly of *M. mutica* by constraining the MP searches to recover only those trees that produce a monophyletic *M. mutica*. The shortest two trees generated by the constraint search are 697 steps long (CI = 0.594; RI = 0.636), 36 steps longer than the unconstrained MP tree. The two-tailed Wilcoxon signed-ranks test (Templeton, 1983) fails to support (P < 0.0001) the monophyly of *M. mutica*. The *mutica* complex is linked to a tenuous *M. leprosa* and *M. caspica* group. Lastly, both MP and Bayesian phylogenetic analyses tentatively place *M. rivulata* sister to a monophyletic clade containing the remaining ingroup taxa.

Genetic Variation. - Our samples of *M. leprosa* from Spain and Morocco, and *M. caspica* from Iran and Bahrain, show no intraspecific haplotype diversity

(Table 1), yet exhibit sizeable morphological variation (Busack and Ernst, 1980). This discrepancy between intraspecific mtDNA diversity and geographic variation seems to be common among turtles (e.g., Lenk et al., 1999; Starkey et al., 2003) and may be related to extensive phenotypic plasticity or the slow rate of molecular evolution in turtles (Avise et al., 1992; Lamb et al., 1994).

In contrast, most interspecific mtDNA variation appears extensive, with uncorrected sequence divergences higher than 8% between a number of ingroup taxa (Table 1). Additionally, the mitochondrial sequence divergences between *M. rivulata* and *M. caspica* (Table 1), formerly considered conspecifics (Fritz and Wischuf, 1997), are equivalent to or greater than the genetic distances observed between other congeneric emydid and batagurid turtles (e.g., Feldman and Parham, 2002; Starkey et al., 2003; Stuart and Parham, 2004). Hence, these mtDNA data, together with the differing shell morphologies, distinct color patterns, and unique habitat preferences of *M. rivulata* and *M. caspica* (Busack and Ernst, 1980), support the recent elevation of *M. rivulata* as a distinct evolutionary lineage independent of *M. caspica* (Fritz and Wischuf, 1997).

Mauremys annamensis, a robust batagurid endemic to central Vietnam, is characterized by extensive axillary buttresses, a massive bridge, a slightly tricarinate and high-domed shell, a vividly striped head and neck, and reverse sexual size-dimorphism (McDowell, 1964; Iverson and McCord, 1994). The taxon is so distinctive it was once placed into its own genus, *Annamemys* Bourret 1939. McDowell (1964) originally demonstrated that *M. annamensis* and *M. mutica* share a number of derived features and Iverson and McCord (1994) subsequently confirmed a close kinship between these taxa with shell measurements. Hence, the close relationship revealed by our mitochondrial genes is not novel. What is surprising, however, is that *M. annamensis* differs from Vietnamese *M. mutica* and our "*M. iversoni*"-like hybrid by only two transitions. Furthermore, this clade shows a roughly 6% uncorrected sequence divergence from topotypic *M. mutica* from Zoushan Island, Zheijung Province, eastern China. In contrast, distantly collected samples of *M. leprosa* and *M. caspica* show no such intraspecific mtDNA variation (Table 1). These data question our ideas of species limits within *Mauremys*. Is *M. annamensis* a distinct species? Does *M. mutica* represent multiple species?

Several potential hypotheses might account for these unexpected results. *M. annamensis* may simply represent a recent species, derived from *M. mutica*, or even a geographical variant of *M. mutica*. The dramatic morphological differences exhibited by *M. annamensis* could reflect intense selection and rapid phenotypic evolution while the minute mitochondrial divergences and paraphyly represent the nature of speciation and unsorted polymorphism. Alternatively, there may be historical or ongoing introgression between *M. annamensis* and Vietnamese *M. mutica*, perhaps facilitated by selection.

Two additional hypotheses involve the possibility of hybridization. While our specimen of *M. annamensis* conforms to the species description, it was acquired from a Chinese turtle farm (Appendix 1) where *M. annamensis* and *M. mutica* are reared together in large numbers (J.F. Parham, pers. obs.). Hence, our *M. annamensis* could be a captive hybrid between *M. annamensis* and *M. mutica*, though we find no morphological characters supporting this notion. Ideally, we would examine the morphology and compare the sequences of a wild-caught *M. annamensis* to our sample, but to our knowledge, no tissued, field-collected vouchers of *M. annamensis* exist in collections; all modern museum specimens of *M. annamensis* have been obtained from either animal markets or the pet trade.

Another possibility is that the Vietnamese *M. mutica* could be hybrid offspring of female *M. annamensis* and male *M. mutica*, accounting for the scant mtDNA differences between Vietnamese *M. mutica* and *M. annamensis* and the sizeable divergences between these samples and topotypic *M. mutica*. Although the "Vietnamese *M. mutica*" are phenotypically similar to typical *M. mutica*, their darker coloration is evocative of *M. annamensis*. Both Barth et al. (2004) and Spinks et al. (2004) found substantial mitochondrial variation between *M. mutica* and *M. annamensis*, but we do not know the provenance or morphology of their samples.

The hybridization of batagurid turtles has lead to other cases of taxonomic confusion (Parham and Shi, 2001; Parham et al., 2001; Shi and Parham, 2001; Wink et al., 2001; Spinks et al., 2004) and cannot be discounted here. Unfortunately, our small sample size prohibits us from effectively evaluating these hypotheses. Clearly a more detailed genetic study is needed to unravel this problem. With our present knowledge, any change in conservation policies for *M. annamensis*, one of the world's most poorly known turtles, would be premature.

Biogeography. - The distribution of *Mauremys* is characterized by a major break between the Zagros Mountains of western Iran (easternmost *M. caspica*) and the Annamite Mountains of central Vietnam (range of *M. annamensis*). This disjunction includes the entire Indian subcontinent (home to a diverse, endemic batagurid fauna), and the inhospitable Tibetan plateau. We suggest that the collision of India into Asia may be the vicariant event responsible for the current distribution of *Mauremys*, as proposed for anguine lizards (Macey et al., 1999). Molecular data are ambiguous on this point.

Given that neither eastern nor western species assemblages appear monophyletic (though a Wilcoxon signed ranks test topology test cannot discount this hypothesis [$P = 0.35$]), the current divergences between the living species may have occurred before the development of the Indo-Tibetan gap. The collision and subsequent uplift of the Tibetan plateau took place in multiple stages between 50 and 10 MYBP (Shackleton and Chang, 1988; Dewey et al., 1989; Windley, 1988). Hervet (2004) attributed some Paleogene (>50 MYBP) European fossils to the stem of *Mauremys*, but did not investigate their relations to east Asian *Mauremys*. In addition to employing additional molecular markers to vouchered museum specimens, the integration of all extant *Mauremys* into analyses of morphological characters and fossil taxa will be necessary to unravel the historical biogeography of this clade of turtles.

Acknowledgments

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Appendix 1.

Specimens used and GenBank Accession numbers for DNA sequence data. Acronyms are: MVZ = Museum of Vertebrate Zoology, Berkeley, California; ROM = Royal Ontario Museum, Toronto, Ontario; AF or AY = GenBank (http://www.ncbi.nlm.gov).

Mauremys annamensis - Purchased in turtle farm in Hainan Province, China, no real locality data; MVZ 238937; AY337338, AY337346. *Mauremys caspica* - Field collected on Bahrain Island, Bahrain; MVZ 230971; AY337339, AY337347. *Mauremys caspica* - Field collected in West Azarbaijan Province, Iran; MVZ 234281; AY337340, AY337348. "*Mauremys iversoni*" - Purchased in turtle farm in Hainan Province, China, no real locality data; MVZ 230475; AF348275, AF34281. *Mauremys japonica* - Pet trade specimen, no locality data; MVZ 234647; AY337341, AY337349. *Mauremys leprosa* - Field collected in Tetouan Province, Morocco; MVZ 178059; AY337342, AY337350. *Mauremys leprosa* - Field collected in Cadiz Province, Spain; MVZ 231989; AY337343, AY337351. *Mauremys mutica* - Field collected in Zoushan Island, Zhejiang Province, China; MVZ 230476; AF348262, AF348278. *Mauremys mutica* - Purchased from a turtle trader in Yen Bai Province, Vietnam; ROM 25613; AF348260, AF348279. *Mauremys mutica* - Purchased from a turtle trader in Yen Bai Province, Vietnam; ROM 25614; AF348261, AF348280. *Mauremys rivulata* - Field-collected in Bursa Province, Turkey; MVZ 230212; AY337344, AY337352. *Mauremys* (=*Ocadia*) *sinensis* - Field-collected in Hainan Province, China; MVZ 230479; AY337345, AY337353. *Mauremys nigricans* - Pet trade specimen, no locality data, MVZ 130463; AF348264, AF348289. *Mauremys reevesii* - Pet trade specimen, no locality data, MVZ 230533; AF348263, AF348288. *Cuora picturata* - Purchased from a turtle trader in Dong Nai Province, Vietnam, ROM 37067; AF348265, AF348292. *Cuora trifasciata* - Pet trade specimen, no locality, MVZ 230636; AF348270, AF348297. *Cuora mouhotii* - Purchased from a turtle trader in Bac Thai Province, ROM 35003; AF348273, AF348286.